

THE C-MYC CODING REGION DETERMINANT-BINDING
PROTEIN (CRD-BP) AND ITS NUCLEIC ACID SEQUENCE

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. provisional
5 application Serial No. 60/077,372 filed March 9, 1998.
Serial No. 60/077,372 is incorporated by reference as if
herein set forth completely.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH
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10 This invention was made with United States
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BACKGROUND OF THE INVENTION

15 The *c-myc* protein is a member of the
helix-loop-helix/leucine zipper (HLH/LZ)¹ family of
transcription factors that forms heterodimers with Max

¹The abbreviations used herein are: HLH/LZ, helix-loop-helix/leucine zipper; AURE, AU-rich element; UTR, untranslated region; CRD, coding region determinant; CRD-BP, coding region determinant-binding protein; DTT, dithiothreitol; EGTA, ethylene glycol bis(2 aminoethyl ether)-N,N' (tetraacetic acid); PMSF, phenylmethyl-sulfonylflouride; S130, post-polysomal supernatant; SDS, sodium dodecyl sulfate; RSW, ribosomal salt wash; PCR, polymerase chain reaction; bp, base pairs; EST, Expressed Sequence Tags; RACE, rapid amplification of cDNA ends; BAC, Bacterial Artificial chromosome; GCG, Genetics Computer Group; IP, immunoprecipitation; mRNP, messenger ribonucleoprotein; hnRNPK, heterogeneous nuclear ribonucleoprotein K; HRP, horseradish peroxidase; HSP-90, heat shock protein-90; MOPS, morpholinepropanesulfonic acid; KH, K homology; ORF, open reading frame; FMR, familial mental retardation; FMRP, FMR RNA-binding protein; hKOC, human KH domain protein overexpressed in human cancer; PAG, polyacrylamide gel; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescent.

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(1-3). In general, trans-activating Myc:Max heterodimers are found in proliferating cells, while trans-repressing Mad:Max heterodimers are found in differentiated cells. The *c-myc* protein level influences cell proliferation, differentiation, and neoplastic transformation, presumably by affecting the balance between Myc:Max and Mad:Max heterodimers (4). When *c-myc* protein is overexpressed or is induced at inappropriate times, this balance is perturbed, and cell proliferation and differentiation are disrupted. For example, *c-myc* overexpression prevents or delays cell differentiation (5, 6). It also blocks serum-starved cells from entering the G₀ phase of the cell cycle and instead induces them to undergo apoptosis (7). *c-myc* overexpression is also implicated in tumor formation in experimental animals and in human patients with Burkitt's lymphoma (8, 9). These and other deleterious consequences of aberrant *c-myc* expression highlight the importance of understanding all aspects of *c-myc* gene regulation.

20 The *c-myc* protein is regulated by phosphorylation,
protein:protein interactions, and changes in its
half-life (10-12). *c-myc* mRNA levels are regulated
transcriptionally and post-transcriptionally, and changes
in *c-myc* mRNA stability can result in large fluctuations
25 in *c-myc* protein levels. The *c-myc* mRNA half-life is
normally only 10 to 20 minutes but can be prolonged 3- to
6-fold when necessary. For example, *c-myc* mRNA is
relatively stable in replicating fetal rodent

hepatocytes, which produce abundant *c-myc* mRNA. It is far less stable in non-growing adult hepatocytes, which contain little or no *c-myc* mRNA (13, 14). However, it is up-regulated and stabilized several-fold when adult
5 hepatocytes replicate following partial hepatectomy (15, 16).

Two *cis*-acting sequence elements in *c-myc* mRNA contribute to its intrinsic instability and perhaps also to its post-transcriptional regulation: an AU-rich
10 element (AURE) in the 3'-untranslated region (3'-UTR) and a 180 nucleotide coding region determinant (CRD). The CRD encodes part of the HLH/LZ domain and is located at the 3' terminus of the mRNA coding region. Four observations indicate how the *c-myc* CRD functions
15 independently of the AURE to affect *c-myc* mRNA expression. (i) *c-myc* mRNA lacking its CRD is more stable than wild-type *c-myc* mRNA (17-20). (ii) The CRD is required for the post-transcriptional down-regulation of *c-myc* mRNA that occurs when cultured myoblasts fuse to
20 form myotubes (20, 21). (iii) Inserting the *c-myc* CRD in frame within the coding region of β -globin mRNA destabilizes the normally very stable β -globin mRNA (22). (iv) The *c-myc* CRD is necessary for up- and
25 down-regulating *c-myc* mRNA levels in transgenic mice undergoing liver regeneration following partial hepatectomy (13, 15, 16, 23-25). In summary, the *c-myc* CRD influences *c-myc* mRNA stability in animals and in cultured cells.

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We have investigated *c-myc* mRNA stability and the function of the CRD using a cell-free mRNA decay system that includes polysomes from cultured cells. The polysomes contain both the substrates (mRNAs) for decay and at least some of the enzymes and co-factors that affect mRNA stability. Polysomes are incubated for different times in an appropriate buffer system, and the decay rates of polysomal mRNAs such as *c-myc* are monitored by hybridization assays. This system reflects many aspects of mRNA decay in intact cells (26-29). For example, mRNAs that are unstable in cells are also relatively unstable *in vitro*; mRNAs that are stable in cells are stable *in vitro* (26). In standard reactions, the polysome-associated *c-myc* mRNA was degraded rapidly in a 3' to 5' direction, perhaps by an exonuclease (29). An alternative decay pathway became activated when the reactions were supplemented with a 180 nucleotide sense strand competitor RNA corresponding to the *c-myc* CRD. This CRD RNA induced endonucleolytic cleavage within the *c-myc* CRD, resulting in an 8-fold destabilization of *c-myc* mRNA (30). These effects seemed to be specific for *c-myc*. Other competitor RNAs did not destabilize *c-myc* mRNA, and *c-myc* CRD competitor RNA did not destabilize other mRNAs tested.

Based on these observations, we hypothesized that a protein was bound to the *c-myc* CRD. We further suggested that this protein shielded the CRD from endonuclease attack, that the CRD competitor RNA titrated the protein

off of the mRNA, and that the unprotected c-myc CRD was then attacked by an endonuclease. Consistent with this model, we detected a protein that binds strongly in vitro to a c-myc CRD ³²P-RNA probe (30). This protein, the c-myc coding region determinant-binding protein (CRD-BP), was subsequently purified to homogeneity (31). We then found that the CRD-BP is developmentally regulated, being expressed in fetal and neonatal rats but not in adult animals (32).

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SUMMARY OF THE INVENTION

In the Examples below, we report the cloning of the mouse CRD-BP cDNA, a novel member of an RNA-binding protein family. We also show that the CRD-BP can bind to ribosomes in vitro and that most of the CRD-BP in cell extracts is located in the cytoplasm and is associated with polysomes and ribosomes. These observations are consistent with a role for the CRD-BP in shielding polysomal c-myc mRNA from endonucleolytic attack, which means that the CRD-BP helps to preserve c-myc mRNA and allows it to be used to make c-MYC protein. We believe that blocking CRD-BP expression might result in the very rapid destruction of c-myc mRNA and subsequent depletion of c-MYC protein from the cell.

We have also shown that the CRD-BP is abundantly expressed in cancer cell lines grown in the laboratory as well as in fetal tissues from rodents (32). In contrast, the CRD-BP is undetectable in tissues from adult rodents

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(32). We believe that these latter observations may be consistent with the idea that the CRD-BP is an oncofetal protein--that is, a protein that is expressed in the fetus and in cancer cells in post-natal life but is not
5 expressed in normal (non-cancerous) tissues in post-natal life. If so, then the CRD-BP should be present in cancer tissues but not in normal tissues in post-natal life.

Specific, restricted expression of the CRD-BP in cancerous tissues could mean that the CRD-BP is a
10 potential diagnostic/prognostic marker for human cancer. Moreover, since the CRD-BP seems to protect c-myc mRNA from being destroyed rapidly, and since c-MYC protein is essential for cell growth, then eliminating the CRD-BP from cancer cells could lead to the cessation of their
15 growth or even to their death.

The present invention is a method of diagnosing the presence or absence of cancer in a human patient comprising the steps of examining patient tissue for the CRD-BP expression levels and comparing that expression
20 level with a control or examining patient serum for antibody against the CRD-BP and comparing that antibody level with that of normal controls (preferably age-matched and sex-matched). Preferably, the control for the CRD-BP expression level in tissues is a non-cancerous
25 tissue from the same source as the test tissue. For example, a breast assay would preferably have a breast tissue control. In a preferred embodiment of the present invention, the cancer is selected from the group

consisting of breast cancer, colon cancer and pancreatic cancer.

In another preferred embodiment of the present invention, the detection of CRD-BP comprises the step of homogenizing biopsy tissue and obtaining a crude protein extract. One would then examine that extract for the CRD-BP level.

The present invention is also a quantitative method of determining the stage of cancer in a human patient comprising the step of examining patient tissues for the CRD-BP expression level and correlating that expression level with the disease prognosis.

The present invention is also a method of inhibiting cancer cell growth comprising the step of eliminating or lowering the level of CRD-BP in the cancerous cells.

It is an advantage of the present invention that a method of diagnosing human cancers is disclosed.

It is another advantage of the present invention that a method of inhibiting cancer cell growth is disclosed.

Other objects, advantages and features of the present invention will become apparent after one of skill in the art has examined the specification, claims and drawings.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Fig. 1. Mouse CRD-BP cDNA and predicted protein sequence (SEQ ID NOs:1 and 2, respectively). Peptide

sequences resembling nuclear localization and nuclear export signals are denoted by the single underline and the overlines, respectively. Peptide sequences resembling the RGG box and the KH domains are denoted by the box and the double underlines, respectively. An asterisk indicates the translation termination site, and the polyadenylation signal is single underlined. We have not demonstrated conclusively that the translation start site indicated in the figure is the correct or the only start site. The 5'-UTR might be incomplete, since the transcription start site has not been mapped.

Fig. 2. CRD-BP alignments with various consensus sequences in RNA binding proteins (SEQ ID NOs:3-30). Shown are alignments of the mouse CRD-BP (mCRD-BP) to the RGG domains (A) (SEQ ID NOs:3-9) nuclear export signals (B) (SEQ ID NOs:10-16), and KH domains (C) (SEQ ID NOs:17-30) of other RNA-binding proteins. Referring to Fig. 2, boxed residues indicate identity with or conservation to the consensus sequence residue. The Genbank accession numbers of the proteins are as follows: hKOC, U97188; hnRNPK, S74678; fibrillarin, X56597; nucleolin, M60858/J05584; FMRP, S65791; Rev, X58781.

Fig. 3. Immunoblotting assay showing co-migration of recombinant and cell derived CRD-BP. Ribosomal salt wash (RSW) was prepared from K562 and NIH/3T3 cell polysomes and from polysomes isolated from reticulocyte transcription/translation reactions programmed with CRD-BP DNA or with vector DNA. Approximately 7.5×10^5

cell equivalents of K562 or NIH/3T3 RSW or 3% of the RSW recovered from a 50 μ l translation reaction were electrophoresed in a 10% SDS-PAG and transferred to a membrane, which was incubated with anti-CRD-BP IgY antibody and then with HRP-conjugated anti-IgY antibody. The signal was developed with Supersignal chemiluminescent reagents. The locations of the CRD-BP and a cross-reacting protein (p85) are indicated. The locations of prestained molecular mass markers are shown on the right in kDa.

Fig. 4. Gel retardation assay showing specific binding of recombinant CRD BP to *c-myc* CRD RNA. (A) RSW was prepared from K562 cell polysomes and from transcription/translation reactions programmed with CRD-BP cDNA, luciferase cDNA (Luc), or vector DNA. Equivalent volumes (2 μ l) of each RSW were incubated with 50,000 cpm of synthetic *c-myc* CRD 32 P-RNA. RNA/protein complexes were separated from free (unbound) probe by electrophoresis in a 6% nondenaturing PAG. "None" indicates a gel retardation reaction to which no protein was added. The positions of CRD-BP/CRD complexes (Bound) and of unbound (Free) RNA are indicated on the left. (B) Competition assay. The indicated RSW was incubated with *c-myc* CRD 32 P-RNA in the presence or absence of buffer (None) or a 200-fold molar excess of unlabeled synthetic *c-myc* CRD RNA or β -Globin RNA. RNA/protein complexes were then separated in a 6% nondenaturing PAG. The

positions of CRD-BP/CRD complexes (Bound) and of unbound (Free) RNA are indicated on the left.

Fig. 5. Co-fractionation of recombinant CRD-BP with reticulocyte ribosomes and ribosomal subunits.

5 Radiolabeled recombinant CRD-BP (filled circles) and luciferase (LUC; unfilled circles) were synthesized in separate reticulocyte translation assays. Each extract was then fractionated by sedimentation through a 20-40% linear sucrose gradient. Equivalent amounts of each
10 gradient fraction were analyzed for radiolabeled protein by electrophoresis in a 10% SDS-PAG and quantitation in the Phosphorimager. The quantity of CRD-BP and luciferase is given in arbitrary units. The locations of ribosomal subunits, monosomes, and polyribosomes were
15 determined by measuring A260 and by electrophoresing a portion of each fraction in an agarose gel, to identify 18S and 28S rRNAs.

Fig. 6. Co-fractionation of endogenous CRD-BP with K562 cell polysomes and lack of CRD-BP in nuclei.

20 Subcellular fractions were prepared from exponentially growing K562 cells (Experimental Procedures). Equal cell equivalents (6×10^5) of each fraction were separated in a 10% SDS-PAG, transferred to a nitrocellulose membrane, and incubated with either (A) anti-CRD-BP IgY or (B)
25 anti-HSP-90 IgG, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. Immunoreactive proteins were visualized using ECL

reagents. The positions of molecular mass markers are indicated on the left in kDa.

Fig. 7. Co-fractionation of the CRD-BP with ribosomal subunits from K562 cells. Polysomes from exponentially growing K562 cells were resuspended in buffer and then incubated in 20 mM EDTA to dissociate ribosomal subunits (60S and 40S) from each other and from mRNP. An aliquot of the subunits was centrifuged in a linear 5-30% sucrose gradient containing EDTA. Fraction 1 is the top of the gradient, fraction 18 is the last gradient fraction, and fraction 19 is the pellet resuspended from the bottom of the centrifuge tube. Panel A: absorbance of each fraction at 260 nm. Panel B: RNA isolated from an aliquot of each fraction was electrophoresed in a 1% agarose gel, which was stained with ethidium bromide and photographed under UV light. The positions of the 28S and 18S rRNAs from the large and small ribosomal subunits, respectively, are noted on the left. Panel C: An aliquot of each fraction was analyzed by immunoblotting using anti-CRD-BP IgY. Immunoreactive proteins were visualized using ECL reagents. The positions of molecular mass markers are indicated in kDa on the left. The CRD-BP and the cross-reacting p85 are noted on the right.

Fig. 8. Co-fractionation of the CRD-BP with 60S ribosomal subunits as determined by immunoprecipitation with anti P-protein antibody. An aliquot of EDTA dissociated K562 cell polysomes was incubated with

anti-P protein antibody (I) or with normal human serum
(N). Antibody-antigen complexes were immunoprecipitated
(IP'd), and IP'd proteins were immunoblotted and analyzed
using anti-P protein IgG (panel A) or anti-CRD-BP IgY
5 (panel B). Immunoreactive proteins were visualized using
ECL reagents. The locations of the P proteins (P_0 , P_1 and
 P_2) and the CRD-BP are indicated on the right. The
positions of prestained molecular mass markers are
indicated in kDa on the left. Heavy chain indicates
10 cross-reactivity with the IgG heavy chain on the
membrane.

DETAILED DESCRIPTION OF THE INVENTION

A. In General

The half-life of *c-myc* mRNA is regulated when cells
15 change their growth rates or differentiate. Two
sequences within the *c-myc* mRNA molecule determine its
half-life, one in the 3'-untranslated region, the other
in the coding region. A cytoplasmic protein, the coding
region determinant-binding protein (CRD-BP), binds in
20 vitro to the *c-myc* coding region stability determinant.

Based on observations using a cell-free mRNA decay
system, we propose that the CRD-BP, when bound to the
mRNA, shields the mRNA from endonucleolytic attack and
thereby prolongs the mRNA half-life. Here we describe
25 the cloning and further characterization of the mouse
CRD-BP, a 577 amino acid protein containing four hnRNP
K-homology domains, an RGG RNA-binding domain, and

nuclear import and export signals. The CRD-BP is similar to a human protein overexpressed in certain human cancers. Recombinant mouse CRD-BP binds specifically to c-myc CRD RNA *in vitro* and reacts with antibody against
5 human CRD-BP. *In vitro* translated CRD BP binds to ribosomes in the absence of c-myc mRNA, and much of the CRD-BP in cell lysates is associated with ribosomes.

We also describe below proposed methods for the present invention. In one embodiment, we propose a method of diagnosing the presence or absence of cancer in a human patient comprising the steps of examining patient tissue for the CRD-BP expression levels and comparing that result with a control sample and/or examining patient serum for antibody against the CRD-BP and comparing that antibody level with that of normal controls (preferably age-matched and sex-matched). Preferably, the control sample for the CRD-BP expression level in tissues is a non-cancerous tissue from the same source. For example, one would compare the CRD-BP levels of a test breast tissue sample with the CRD-BP levels of breast tissue known to be non-cancerous.

This examination may take the form of examining a crude protein extract for the CRD-BP level, preferably by two antibody sandwich assay, antigen competition assay, antibody capture assay, or by immunoblotting of the crude protein extract with an antibody to CRD-BP. One may also examine the cells in the tissue samples directly for the presence or absence of CRD-BP via immununological methods

involving probing a tissue section with an antibody to CRD-BP or via *in situ* hybridization methods involving probing a tissue section with a nucleic acid probe specific for the CRD-BP.

5 In another embodiment, the present invention is a method of determining cancer disease prognosis. One would examine the CRD-BP expression levels in a patient tissue sample and correlate these CRD-BP levels with disease prognosis.

10 The present invention is also the use of CRD-BP in immunological assays to identify and quantify anti-CRD-BP antibodies in patient sera. Preferably, one would use recombinant CRD-BP in standard immunological assays. The present invention is also the use of anti-CRD-BP
15 antibodies to identify and quantify the CRD-BP itself in serum from cancer patients.

We expect to find that certain expression levels of CRD-BP can be directly correlated with, and are therefore predictive of, certain cancers.

20 We also propose a method of inhibiting cancer cell growth by eliminating or lowering the level of CRD-BP from the cancerous cells. Preferably, this method is either by providing the cell with competitor RNA or by use of an inhibitor that blocks CRD-BP binding to the c-
25 *myc* mRNA CRD.

By "CRD-BP" we preferably mean the protein as described herein at SEQ ID NO:2 and in Ref. 30, 31 and 32 below.

One typical way to obtain a CRD-BP antibody would be to make large amounts of recombinant CRD-BP in either bacterial cells, yeast cells or baculovirus-infected insect cells. This protein is then injected into rabbits, sheep or goats to make a polyclonal antibody. Epitope-specific antibodies can also be made by using synthetic peptides (8-15 amino acids) as the immunogen. These are routine techniques known to those of skill in the art.

10 B. Detecting the CRD-BP in Clinical Samples

We have hypothesized that the CRD-BP might be an oncofetal protein. This hypothesis is based on our findings that the CRD-BP is expressed in fetal rat tissues but not in normal adult rat tissues. It is also expressed in tissue culture cell lines, which are neoplastic.

We show below in the Examples that the CRD-BP is significantly more abundant in tumor tissue than in a normal adult tissue. Therefore, we envision that the presence of the CRD-BP in biopsy specimens indicates that the specimens contain tumor cells. We envision that the presence of the CRD-BP is indicative of neoplasia and would be a prognostic and diagnostic indicator.

There are many possible CRD-BP detection schemes. The best scheme will depend on the following variables: the amount of CRD-BP expressed in the tumor tissue, the specificity and avidity of the antibodies for the CRD-BP, and the extent of cross-reactivity of the antibodies with

other proteins besides the CRD-BP. Below is an outline of several possible detection schemes.

It is probably best to ensure that the antibodies are specific for the CRD-BP. We can do so by making antibodies against CRD-BP peptides or by using monoclonal antibodies that, on Western blots, react only with the CRD-BP and not with any other cellular proteins.

1. Detection of the CRD-BP using protein extracts:
Biopsy tissue would be homogenized and a crude
protein extract would be prepared (Proposed).
- a. Exemplary Detection schemes in which antigen or antibody is bound to a solid support.
- i. Two antibody sandwich assay: A monoclonal antibody recognizing one CRD-BP epitope is bound to a solid support such as a microtiter well. The sandwich assay would also work with two polyclonal antibodies, as long as each antibody was against a different epitope in the CRD-BP. An extract of the tissue is added, and CRD-BP in the extract is permitted to bind to the antibody. Then a second monoclonal recognizing a different CRD-BP epitope is added. The second antibody can be labeled with ^{125}I or ^3H . Then, the amount of labeled antibody bound will provide a measure of the amount of CRD-BP attached to the first antibody.

Alternatively, a tagged secondary antibody can be used for quantitation. This secondary antibody can be tagged with an enzyme such as horseradish peroxidase or with a probe such as biotin. The amount of bound secondary antibody is then detected by standard assays

and is a measure of the amount of CRD-BP in the tissue extract.

ii. Antigen competition assay: Anti-CRD-BP antibody is bound to a solid support such as a microtiter well. The tissue extract is then mixed with purified, radiolabeled CRD-BP. If the tissue contains sufficient CRD-BP, this CRD-BP will compete with the labeled CRD-BP for binding to limiting antibody. Thus, the amount of CRD-BP in the extract will be inversely proportional to the amount of labeled CRD-BP bound to the microtiter well. We know the nucleic acid sequence of the human CRD-BP coding region. Therefore, we should be able to prepare highly purified, radiolabeled CRD-BP using bacteria, yeast, or insect cells.

Prokipcak, et al. (ref. 31) discloses one method of purification of CRD-BP. We also envision an easier purification scheme that exploits added epitopes. Instead of making unmodified CRD-BP in bacteria, yeast, or baculovirus-infected cells, we could use molecular techniques to design a CRD-BP complementary DNA that would generate an "epitope-tagged" CRD-BP. We could express the tagged CRD-BP in cells and then purify the CRD-BP in a single affinity step that exploits the tag to separate CRD-BP from all the other cell proteins.

iii. Antibody capture assay: The tissue extract is bound to a microtiter well. Antibody is added, and the amount of antibody bound is determined. The antibody can be labeled or unlabeled. If it is

unlabeled, the amount bound is determined indirectly, using anti-antibody antibodies and detecting them by peroxidase or biotin labeling, as described above.

5 b. Exemplary Detection of the CRD-BP by
 Immunoblotting (Western Blotting)

Tissue extract is electrophoresed in a denaturing gel, and the proteins are transferred to a nitrocellulose or PVDF membrane. The membrane is then probed with anti-CRD-BP antibody, and the amount of antibody bound is
10 determined by any of a variety of detection techniques using tagged anti-antibody antibodies. The disadvantage of Western blotting is that it is more time-consuming than assays in which the extract protein or the antibody is bound to a solid support. The advantage is that
15 specific interactions are more readily discerned, and artifacts are eliminated. The presence of the CRD-BP in a Western blot is indicated by a band at the -68 kilodalton region of the gel.

We envision that the assay might be simplified to
20 the point that a dipstick or colorimetric assay could be used.

2. Detection of CRD-BP in cells by immunohistochemistry

25 In a typical method, the biopsy tissue is cut into a thin section and fixed and then analyzed using standard immunohistochemical techniques. The detection system will depend on the amount of CRD-BP in the tissue. Although this technique is more time-consuming than
30 techniques using tissue extracts, immunohistochemistry

can identify rare abnormal cells. For example, a biopsy specimen might contain primarily normal cells with only small patches of neoplastic cells. If the neoplastic cells express the CRD-BP, then they might be visualized
5 by immunohistochemistry using CRD-BP-specific antibodies.

3. Detection of CRD-BP in cells by in situ hybridization

In a typical method, the biopsy tissue is cut into a thin section and fixed and then analyzed using standard
10 in situ hybridization techniques with a CRD-BP DNA or RNA probe. As is the case with immunohistochemistry, an advantage of the in situ hybridization technique is the ability to detect rare cancerous cells in the midst of a majority of normal cells.

15 C. Detecting CRD-BP or CRD-BP Antibodies in Patient Sera

The CRD-BP is a cytoplasmic protein. Therefore, it should not be exposed to immune cells under most conditions. However, if it is overexpressed in human
20 tumor cells, and if these cells undergo lysis or the protein for whatever reason leaks out of the cells, the CRD-BP itself might be detected in patient serum, and/or antibodies to the CRD-BP might arise in patients with tumors. Detecting the CRD-BP or such antibodies in a
25 small amount of patient serum would then provide a rapid and convenient screen for cancer. The previous section outlined methods for detecting the CRD-BP. Strategies to detect anti-CRD-BP antibodies might exploit techniques similar to those for detecting the CRD-BP itself in

extracts from biopsy material. There are many ways for detecting antibodies. Some of the techniques that would be suitable for detecting anti-CRD-BP antibodies in patient serum are summarized below.

5 i. Two Antibody Sandwich Assay

 The CRD-BP itself will be made in bacterial, yeast or insect cells using standard techniques. This recombinant CRD-BP will then be bound to a solid support such as a microtiter well. Patient serum is added, and
10 anti-CRD-BP antibody in the serum is permitted to bind to the CRD-BP. The plates are then washed extensively, and a second anti-human serum is added. The second antibody can be labeled with 125I or 3H or with a fluorescent tag. Then, the amount of labeled antibody bound will provide a
15 measure of the amount of anti-CRD-BP antibody attached to the recombinant CRD-BP on the plate. Alternatively, a tagged secondary antibody can be used for quantitation. This secondary antibody can be tagged with an enzyme such as horseradish peroxidase or with a probe such as biotin.
20 The amount of bound secondary antibody is then detected by standard assays and is a measure of the amount of anti-CRD-BP antibody in the serum of the patient.

 ii. Antigen Capture Assay

 Serum from the patient is attached to a solid
25 support such as a microtiter well. Then radiolabeled, recombinant CRD-BP is added. Unbound CRD-BP is washed off of the plate, and the amount of bound antigen is measured. The radiolabeled CRD-BP could be labeled in

vivo in bacteria or yeast using 35S or could be radioiodinated *in vitro*.

D. Treatment of cancer by eliminating the CRD-BP from the cancer cells

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The basic idea of the present invention is primarily based on two notions: that the CRD-BP stabilizes c-myc mRNA in cells and that the CRD-BP is expressed post-natally in tumor cells but not in normal cells. As a result, c-myc mRNA is overexpressed or inappropriately expressed in tumor cells. If the CRD-BP could be eliminated, then c-myc mRNA would be destabilized. If c-myc mRNA were essential for growth or viability of the tumor cells, then the tumor cells would stop growing or die. Selectivity would be assured if the CRD-BP were expressed more abundantly in tumor cells.

10

Two approaches are preferred for interfering with the interaction of the CRD-BP with c-myc mRNA:

1. Genetic engineering

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The way we destabilized c-myc mRNA in our cell-free mRNA decay system was to add excess competitor RNA to the reactions. The RNA contains the 180 nucleotides of the c-myc mRNA coding region determinant (CRD). The competitor RNA is thought to titrate the CRD-BP from c-myc mRNA. As a result, the CRD of c-myc mRNA is not shielded by the CRD-BP, and the mRNA is rapidly degraded by a ribonuclease.

25

In order to exploit a similar strategy in intact cells, it would be necessary to apply the techniques of

genetic engineering to overexpress c-myc mRNA CRD RNA in the affected tissue or organ. One might introduce DNA capable of expressing the CRD competitor RNA in the tissue or organ. Alternatively, it might be feasible to introduce a ribonuclease-resistant, long-lasting form of CRD RNA itself. It is important to note that specificity would be achieved if the target cancer cells were expressing the CRD-BP, while non-cancer cells did not express it. Under these conditions, the competitor CRD RNA would have a deleterious effect only on the cancer cells.

2. Use of an inhibitor that blocks CRD-BP binding to the c-myc mRNA CRD

We presume that the CRD-BP folds in such a way that it is able to recognize a particular segment of c-myc mRNA, namely, the CRD RNA segment. One could design peptide or nucleic acid analogues or other compounds that bind to the CRD-BP so as to inhibit its ability to interact with c-myc mRNA in cells. This is similar to strategies that are being considered by pharmaceutical companies hoping to design antiviral compounds capable of entering cells and interacting with viral-derived proteins and nucleic acids. The protease inhibitors used in HIV-infected patients are an example of a pharmaceutical agent directed against a specific viral-encoded product.

EXAMPLES

A. Experimental Procedures

Cell lines and preparation of subcellular fractions.

All cell lines were obtained from the American Type
5 Culture Collection (Rockville, MD). K562 human
erythroleukemia cells were cultured in RPMI-1640 medium
containing 10% calf serum plus a penicillin/streptomycin
mix. NIH/3T3 cells were grown in DMEM (4.5 g/L glucose)
containing 10% calf serum and antibiotics. All
10 antibiotics and sera were from Gibco/BRL Life
Technologies.

Subcellular fractions were prepared as follows. All
steps following cell harvesting were at 4°C. Cells were
grown in 1 liter spinner flasks to a density of $3-5 \times 10^5$
15 cells/ml. They were harvested, collected by low speed
centrifugation, and washed 3 times with cold F12 medium
without serum. The cell pellet was resuspended at a
density of 1.5×10^7 cells/ml in Buffer A (1 mM potassium
acetate, 1.5 mM magnesium acetate, 2 mM DTT, 10 mM
20 Tris-Cl, pH 7.4) containing 100 mM EGTA, 100 mg/ml PMSF,
and 2 mg/ml each of aprotinin, leupeptin, and pepstatin A
(all from Sigma). The cells were lysed with 30-40
strokes of a Dounce homogenizer, and the lysate was
centrifuged for 10 minutes at 20,000 x g to pellet nuclei
25 and other organelles. The supernatant (S20) was layered
over a cushion of 30% (w/v) sucrose dissolved in Buffer A
and was centrifuged for 2.5 hours at 130,000 x g to
pellet polysomes. The supernatant (S130) above the

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sucrose cushion was harvested, and the polysomal pellet was resuspended in Buffer A containing PMSF, leupeptin, pepstatin A, and aprotinin. The S20 pellet (crude nuclei) was washed once in Buffer A and centrifuged, and the nuclear wash material in the supernatant was harvested and saved. The pelleted, washed nuclei were then resuspended in 300 μ l of Buffer B (1.5 mM MgCl₂, 140 mM NaCl, 20% glycerol, 10 mM Tris-Cl, pH 8.0) and lysed by adding 2.7 ml of Buffer C (5.0 % SDS, 10% glycerol, 5% β -mercaptoethanol, 62.5 mM Tris-Cl, pH 6.8). The extract was then passed 10 times through an 18-gauge needle and boiled for 15 minutes. To isolate ribosomal salt wash (RSW) from either tissue culture cells or reticulocyte translation reactions, an aliquot of polysomes was incubated for 20 minutes at 4°C with 1 M NaCl in buffer A, followed by centrifugation for 2.5 hours at 130,000 x g to re-pellet the salt washed polysomes (26). Glycerol was added to 10% to the supernatant (RSW) above the sucrose cushion, and the salt-washed polysomes were resuspended in Buffer A containing the protease inhibitors. All fractions were stored at -70°C.

Protein purification and microsequencing. The human c-myc CRD-BP was purified from K562 cell RSW as described (31). Two independent preparations of CRD-BP from different RSW isolates were microsequenced for this study. The first sequence was determined at the Protein Sequence and Peptide Synthesis Facility of the University of Wisconsin Biotechnology Center (Madison, WI). The

second sequence was distinct from the first, did not overlap, and was determined at the Keck Laboratories, Yale University (New Haven, CT). The second sequence was used for preparing PCR primers.

5 Cloning of mouse CRD-BP cDNA.

1. CRD-BP cDNA cloning. We first prepared a human CRD-BP cDNA and used its sequence to identify mouse CRD-BP cDNA. DNA oligomers were synthesized by the Nucleic Acid Sequence and Oligomer Synthesis Facility of
10 the University of Wisconsin Biotechnology Center (Madison, WI) or by GIBCO-BRL Life Technologies (Grand Island, NY). A K562 (human) cell cDNA lambda library (Clontech, Palo Alto, CA) was first screened by degenerate PCR in order to amplify a 45 bp DNA sequence
15 based on the 15 amino acids of the second CRD-BP peptide sequence. The following primers were used:
5'-GTBAAYGARYTBCARAA-3' (coding) (SEQ ID NO:31) and
5'-GGVACVACVACYTCDGC-3' (non-coding) (SEQ ID NO:32). The conditions were 30 cycles, 94°C for 30 seconds, 45°C for
20 30 seconds, 72°C for 1 minute, AMPLITAQ DNA Polymerase (Perkin Elmer). PCR products from this and subsequent reactions were subcloned directly into pT7-Blue (Novagen, Madison, WI) for sequencing, which was performed by PCR using the ABI Prism AmpliTaq FS Dye Terminator Reaction
25 Kit (Applied Biosystems, Inc.) according to the manufacturer's recommendations. A 45 bp product encoding the expected 15 amino acid sequence was isolated in this way. The same cDNA library was then used for non

degenerate PCR with a CRD-BP-specific coding primer from
the middle of the 45 bp sequence
(5'-GCTGCCGTCAAATTCTG-3') (SEQ ID NO:33) plus a
lambda-specific primer (5'-TCGACGGTTTCCATATG-3') (SEQ
5 ID NO:34) under the following conditions: 30 cycles,
94°C for 30 seconds, 50°C for 30 seconds, 72°C for 3
minutes, AMPLITAQ DNA Polymerase. This step generated a
227 bp cDNA. The same library was then plated,
transferred in duplicate to nitrocellulose filters, and
10 screened by hybridization with the 227 bp ³²P-DNA as
probe. This step generated a 1069 bp partial human
CRD-BP cDNA with an open reading frame encoding both of
the peptides obtained by sequencing purified CRD-BP.
This cDNA did not contain the 5' part of the coding
15 region, the 5'-UTR, or most of the 3'-UTR.

To complete the cloning of the 3' terminal region ,
3' rapid amplification of cDNA ends (3'-RACE) was
performed. Oligomer Not(dT) (5'-AACCCGGCTCGAGCGGCCGC
TTTTTTTTTTTTTTTTTTT-3') (SEQ ID NO:35) and Superscript II
20 (GIBCO-BRL) were used according to the manufacturer's
recommendations to reverse transcribe 0.5 µg of K562 cell
poly(A)+ mRNA. The cDNA template was then amplified
using VENT DNA Polymerase (New England Biolabs) with
oligomers CRD-BP1 (5' ACGGCAGCTGAGGTGGTAGTACC-3') (SEQ ID
25 NO:36) and NotAdaptmer (5'-AACCCGGCTCGAGCGGCCGCT-3') (SEQ
ID NO:37) as 5' and 3' primers, respectively. Conditions
were 1 cycle of 94°C for 1 minute, followed by 35 cycles

of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1.5 minutes.

2. Cloning of mouse CRD-BP cDNA. The partial human CRD-BP cDNA generated as described above was used to identify mouse CRD-BP cDNAs in the EST Database using the NCBI Blast Program. The larger of the two EST's, AA073514, was obtained from Genome Systems, Inc. (St. Louis, MO) and was sequenced. The amino acid sequence it encoded was 99% identical to that of our human CRD-BP, indicating that it corresponded to the mouse CRD-BP. It contained the entire 3'-UTR and most of the coding region. To extend the 5' sequence, 5'- RACE was performed on a 17 day mouse embryo Marathon-Ready cDNA Library (Clontech) using ADVANTAGE KlenTaq DNA Polymerase (Clontech) according to the manufacturer's instructions. In primary reactions, "touchdown PCR" was performed with oligomers AP1 (Clontech) and CRD-BP2 (5'-AGGTTCCGTCCTTCCTTGCCAATG-3') (SEQ ID NO:38) as 5' and 3' primers, respectively. Conditions were 1 cycle of 94°C for 1 minutes, 5 cycles of 94°C for 10 seconds, 72°C for 7.5 minutes, 5 cycles of 94°C for 10 seconds, 70°C for 7.5 minutes, 20 cycles of 94°C for 10 seconds, 68°C for 7.5 minutes, 10 cycles of 94°C for 10 seconds, 60°C for 20 seconds, 68°C for 7.5 minutes. DNA bands were excised from a 1% agarose gel, and secondary PCR was performed with them using nested 5' and 3' primers [oligomers AP2 (Clontech) and CRD-BP3 (5'-AACTTCATCTGCCGTTTTGG 5') (SEQ ID NO:39), respectively]. Conditions were 1 cycle of

94°C for 1 minutes, followed by 25 cycles of 94°C for 15
second, 60°C for 30 seconds, 68°C for 5 minutes. Since
the resulting clone did not contain the translation start
site or any 5'-UTR, a mouse BAC library was screened for
5 the CRD BP gene by PCR with primers CRD-BP4
(5'-CATCAACTGGAGAACCATG-3') (SEQ ID NO:40) and CRD-BP5
(5'-GACTGCGTCTGTTTTGTGATG-3') (SEQ ID NO:41). A BAC
clone containing the mouse CRD-BP gene was obtained from
Genome Systems. The remainder of the coding region and
10 at least part of the 5'UTR was sequenced from this BAC
clone using oligomer CRD BP6 (5'-CTGTAGGAGATCTTGTGCTC-3')
(SEQ ID NO:42) as primer. Sequence comparisons were
generated using the Genetics Computer Group (GCG) Bestfit
and Gap algorithms. Theoretical translations were made
15 with the GCG Translate program.

In vitro translation of mouse CRD-BP. A portion of
the mouse CRD-BP cDNA was subcloned into pSPUTK
(Stratagene, La Jolla, CA) to create the translation
clone pSPUTK-CRD-BP as follows: A single base mutation
20 (underlined) was made in the 5' primer
(5'CGCACCGCCACCATGGACAAGCTTTACATCGG-3') (SEQ ID NO:43) to
generate an NcoI site for subcloning. The mutation
changes an asparagine to an aspartic acid. The 3' primer
(5'-ACTGGGATCTGACCCATCCT-3') (SEQ ID NO:44) was from the
25 CRD-BP 3'-UTR. Conditions were 1 cycle of 94°C for 1
minute, followed by 25 cycles of 94°C for 30 seconds, 55°C
for 30 seconds, 68°C for 3 minutes. pSPUTK-CRD-BP,
pSPUTK-Luciferase, or pSPUTK vector templates were

transcribed and translated using the TnT® Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's instructions.

Immunoprecipitation, immunoblotting, and gel

5 retardation assays. Immunoprecipitation (IP) of 60S ribosomal subunits was performed essentially as previously described (33). Briefly, human anti-P protein serum (Immunovision) or normal human serum was conjugated to Protein G-Plus Sepharose beads (Oncogene Science).

10 The anti-P protein serum recognizes three large ribosomal subunit proteins (P_0 -38 kDa, P_1 -19 kDa, P_2 -17 kDa; ref. 34). K562 polysomes were dissociated into mRNP and ribosomal subunits by incubation with 20 mM EDTA at 4°C for 20 minutes. Protein G-Plus Sepharose-conjugated
15 antibodies were then incubated with 10 μ l of the dissociated polysomes in IP buffer (100 mM KCl, 5 mM EDTA, 1 mM DTT, 0.5% Triton X-100, 100 μ g/ml PMSF, 0.5% aprotinin, and 2 μ g/ml each leupeptin and pepstatin A, 10 mM HEPES, pH 7.3) for 16 hours at 4°C with gentle mixing.
20 The beads were washed three times for 20 minutes each at 4°C in IP buffer. Bound proteins were eluted by resuspending the beads in Buffer D (2.3% SDS, 10% glycerol, 62.5 mM Tris-Cl, pH 6.8) and incubating the beads at 95°C for 5 minutes.

25 Immunoblotting was performed as previously described (32). For CRD-BP, the primary antibody was a chicken anti-CRD-BP IgY raised against the purified human protein (31, 32), and the secondary detection antibody was

horseradish peroxidase (HRP) conjugated rabbit anti-chicken IgY (Promega). For the ribosomal P proteins, human anti-P protein serum (see above) was the primary antibody, and the secondary detection antibody was HRP-conjugated goat anti-human IgG (Promega). For heat shock protein-90 (HSP 90), the primary antibody was a rabbit anti-mouse HSP-90 polyclonal IgG (a kind gift from Dr. Alan Poland), and the secondary detection antibody was HRP-conjugated goat anti-rabbit IgG (Sigma). Blots were developed by enhanced chemiluminescence (ECL) using either standard (Amersham) or Supersignal ULTRA (Pierce) reagents. Distinct bands were not detected with preimmune antibodies, normal human serum, or secondary antibodies alone (data not shown). Where noted, blots were stripped for 30 minutes at 50°C in 2% SDS, 100 mM β -mercaptoethanol, 50 mM K₂HPO₄, pH 6.8 and were then washed extensively in buffer containing 5% nonfat dry milk to remove SDS and β -mercaptoethanol. Gel retardation assays were performed as previously described (31, 32).

Sucrose gradient centrifugation and ribosomal RNA analysis. All procedures were performed at 4°C. For analyzing the CRD-BP association with ribosomal subunits, an aliquot of K562 cell polysomes (50 μ l) or cytoplasmic lysate (S20; 150 μ l) was brought to a final concentration of 20 mM EDTA. The material was mixed gently, left on ice for 20 minutes, layered over a 10 ml linear 5-30% sucrose gradient in Buffer E (100 mM KCl, 10 mM potassium

acetate, 5 mM EDTA, 1 mM DTT, 5 mM HEPES, pH 7.3) (33),
and centrifuged in a Beckman SW41.1 rotor for 4 hours at
4°C, 38,000 rpm (178,000 x g). Following centrifugation,
500 µl fractions were pipetted sequentially from the top
5 of the gradient. The pellet at the bottom of the tube
was resuspended in 500 µl of Buffer E containing 5%
sucrose. Proteins were precipitated with methanol and
chloroform prior to immunoblotting. RNA from each
fraction was isolated using TRIzol reagent (Gibco/BRL)
10 following the manufacturer's directions and was
electrophoresed in a 1% agarose gel containing 10 mM
sodium acetate, 1 mM EDTA, 40 mM MOPS, pH 7.0. Ribosomal
RNA bands were visualized by staining with ethidium
bromide (0.05 µg/ml).

15 Recombinant, 35S-labeled CRD-BP or luciferase was
synthesized in reticulocyte extracts and analyzed by
sucrose gradient centrifugation essentially as previously
described (35) with slight modifications. The reactions
(100 µl) were chilled on ice, layered over a 4 ml linear
20 20-40% sucrose gradient containing 25 mM potassium
acetate, 1.5 mM magnesium acetate, 1 mM DTT, 20 mM
Tris-Cl, pH 7.2, and centrifuged in a Beckman SW60 rotor
for 5 hours at 4°C, 133,000 x g. Fractions were pipetted
sequentially from the top of the gradient, and 5 µl of
25 each were electrophoresed in a 10% SDS-PAG. Full length
CRD-BP and luciferase protein were quantified by
PhosphorImager analysis using the ImageQuant program
(Molecular Dynamics). Ribosomal RNA from each fraction

was extracted, electrophoresed in a 1% agarose gel, and visualized by staining with ethidium bromide.

B. Results

Cloning the cDNA encoding the CRD-BP, a novel
5 KH-domain RNA binding protein. Two preparations of highly purified CRD-BP were isolated from human K562 cell polysomes in separate experiments. Each preparation was microsequenced, and each gave a different, nonoverlapping sequence, which was P-A-Q-V-G-A-I-Q/I-G-k/r-I/K-Y/G-
10 Q-X-i/l-k (SEQ ID NO:45) from the first and -N-E-L-Q-N-L-T-A-A-E-V-V-V-P (SEQ ID NO:46) from the second. Lower case letters indicate residues of less confidence than upper case letters. A K562 cDNA library was then screened by PCR using degenerate primers based
15 on the amino and carboxy termini of the second peptide (Experimental Procedures). A 45 bp product was generated, subcloned, sequenced, and found to encode the second amino acid sequence. Subsequent PCR amplification and library screening identified a 1069 bp partial human
20 cDNA containing an open reading frame (ORF) that included both peptide sequences obtained by microsequencing.

In order to continue our analysis of the properties and developmental regulation of the mouse CRD-BP, we then exploited the human cDNA sequence to isolate a putative
25 mouse CRD-BP cDNA (Experimental Procedures). A clone containing at least a portion of the 5'-UTR, a complete coding region, and a complete 3'-UTR was obtained and sequenced (Fig.1). Two in-frame AUG start codons are

present near the 5' terminus of the cDNA. We have tentatively designated the downstream AUG as the translation start site, because it is embedded within a sequence that is preferred as a translation start signal (36). In contrast, the upstream AUG is not within a preferred translation start motif.

The predicted sequence of the murine cDNA contains several KH domains and an RGG box, which are characteristic motifs found in some RNA-binding proteins. There are four KH domains arranged as two pairs of repeats (Fig. 1, double underlines). Each repeat pair is separated by approximately 30 residues, and the two pairs of repeats are separated by 78 residues. The putative RGG box (boxed) is located upstream of the KH domains. There are two putative nuclear export signals (overlined). One is similar to that found in the FMR RNA-binding protein (FMRP), which is associated with familial mental retardation (37-39). The other is similar to that in the HIV Rev protein. There is also a putative nuclear localization signal (underlined).

The RGG, nuclear export, and KH domain regions of the CRD-BP are similar to those found in several other RNA-binding proteins (Fig. 2). Moreover, the human and murine CRD-BP sequences are similar to a human cDNA called hKOC, an acronym for human KH domain protein overexpressed in human cancer (Fig. 2). The hKOC open reading frame encodes a protein of unknown function that was cloned on the basis of its overexpression in human

pancreatic cancer tissue (40). The mouse CRD-BP coding region is 88.8% and 99.1% identical to the coding region of the human CRD-BP at the nucleic acid and protein sequence levels, respectively. For comparison, mouse
5 CRD-BP is 66.6% and 74.0% identical to the hKOC coding region at the nucleic acid and protein levels, respectively. Based on these comparisons and on the data presented below, we conclude that our cDNA encodes CRD-BP and is not the mouse homologue of human KOC. Additional
10 evidence (presented below) suggests that the CRD-BP and hKOC are members of a new subfamily of KH domain containing RNA-binding proteins.

Comparison of in vitro synthesized CRD-BP with cell-derived CRD BP. To determine whether our murine
15 cDNA clone encoded full-length CRD-BP with the expected properties of a c-myc mRNA-binding protein, we synthesized the protein in vitro and analyzed it by immunoblotting and gel retardation assays. Reticulocyte transcription/translation reactions were programmed with
20 CRD-BP cDNA subcloned into a pSPUTK vector. The CRD-BP sequences in the subclone began with the AUG denoted as the translation start site in Fig. 1. This subclone did not contain the upstream, in-frame AUG. The translation extract was fractionated by SDS-PAGE and analyzed by
25 immunoblotting with anti-CRD-BP antibody. A protein of ~68 kDa from the cDNA translation was recognized by anti-CRD-BP antibody and migrated close to the positions of authentic CRD-BP from human (K562) and mouse (NIH/3T3)

cells (Fig. 3, lanes 1-3). An immunoreactive band was not observed in control lanes containing extract programmed with the pSPUTK vector (Fig. 3, lane 4) or with luciferase cDNA (data not shown), indicating that the antibody specifically detected CRD-BP and not an endogenous reticulocyte protein. Therefore, our cDNA encodes CRD-BP. The cross-reacting band (p85) seen in the K562 and NIH/3T3 RSW lanes is a protein observed previously (32). Its identity and function are unknown. p85 does not bind c-myc CRD RNA (32), and it localizes to different subcellular fractions when compared to CRD-BP (see below).

Gel retardation assays were performed to determine if recombinant CRD-BP could bind specifically to c-myc CRD RNA. In preliminary experiments, we noted that most of the recombinant CRD-BP co-fractionated with reticulocyte ribosomes (see below). Therefore, the gel retardation assays were performed using RSW from cells or from reticulocyte translation reactions. RSW's were incubated with c-myc CRD ³²P-RNA, and RNA/protein complexes were resolved from free ³²P-RNA by non-denaturing gel electrophoresis. An RNA/protein complex was observed with protein from K562 cells and from the translation extract programmed with CRD-BP cDNA (Fig. 4A, lanes 1 and 2, respectively). These complexes migrated to similar or identical positions in the gel. An RNA/protein complex was not observed with protein from the luciferase (Luc), Vector, or no mRNA (None) control

reactions (Fig. 4A, lanes 3-5). Therefore, *in vitro* synthesized CRD-BP, like its cell-derived counterpart, associates with *c-myc* CRD RNA *in vitro*.

Previous work had shown that cell-derived CRD-BP did not bind to other RNAs we tested, suggesting that it had considerable specificity for *c-myc* CRD RNA (30, 31). A competition assay was performed to determine if recombinant CRD-BP exhibited similar specificity. RNA-protein binding reactions contained *c-myc* CRD ³²P-RNA as probe plus RSW as a protein source. Reactions were supplemented with no competitor RNA or with a 200-fold molar excess of either unlabeled *c-myc* CRD RNA or β -globin RNA. The CRD BP/CRD ³²P-RNA complex was competed by excess unlabeled CRD RNA but not by β -globin RNA (Fig. 4B). This result further confirms that this cDNA encodes functional *c-myc* CRD-BP.

Co-fractionation of recombinant CRD-BP with ribosomes in reticulocyte extracts. As noted above, preliminary experiments had indicated that a large percentage of recombinant CRD-BP co-sedimented with reticulocyte polysomes. It was important to confirm this finding, because reticulocytes contain no *c-myc* mRNA as measured by Northern blotting. Therefore, it was possible that the CRD-BP, like the FMRP (33), has an affinity for ribosomes even in the absence of what we believe to be its natural mRNA ligand. ³⁵S-Labeled CRD-BP and luciferase were synthesized in reticulocyte extracts, and each extract was sedimented in a sucrose

gradient. Fractions were collected and assayed for ribosome content by gel electrophoresis and for protein by gel electrophoresis and PhosphorImager analysis.

Whereas all of the luciferase sedimented near the top of the gradient (Fig. 5, unfilled circles), greater than 95% of the CRD-BP co sedimented with monosomes and ribosomal subunits (filled circles). Therefore, the CRD BP can bind *in vitro* to ribosomes and ribosomal subunits in the absence of *c-myc* mRNA.

10 Localization of CRD-BP to the cytoplasm and
co-fractionation with ribosomes and ribosomal subunits.

The CRD-BP is located primarily in the cytoplasmic fraction of K562 cell extracts, and much of it is associated with polysomes (ref. 31 and data not shown).

15 This observation is consistent with its putative role as an mRNA-binding protein. However, the amount of CRD-BP per K562 cell exceeds the amount of *c-myc* mRNA by at least 1000-fold (31). Several factors could account for the "excess" CRD-BP in these cells: i) The CRD-BP might
20 be associated with other mRNAs besides *c-myc*. ii) A portion of it might associate with ribosomes and/or ribosomal subunits, as is the case with FMRP (33). An association between the CRD-BP and ribosomes in cells would be consistent with the association of newly
25 synthesized CRD-BP with reticulocyte ribosomes (Fig. 5). Experiments are in progress to determine whether the CRD-BP is bound to *c-myc* mRNA in cells. To determine how much of it co-fractionates with cell ribosomes and

ribosomal subunits and how much, if any, co-fractionates with nuclei, exponentially growing K562 cells were harvested, lysed, and separated into 6 fractions (Experimental Procedures). Equal cell equivalents of each fraction were analyzed by immunoblotting with an anti-CRD-BP antibody. At least 95% of the total cell CRD-BP was in the polysome fraction, and greater than 90% of this CRD-BP was eluted in the one molar salt wash (Fig. 6A, RSW). Little or no CRD-BP was detected in fractions containing nuclei or post-polysomal supernatant (Fig. 6A, Nuclei and S130, respectively). The absence of CRD-BP in these fractions could not be explained by indiscriminate proteolysis during sample preparation, because HSP-90 was detected in all of the fractions (Fig. 6B). Some p85 was detected in both the nuclear and polysomal fractions. This result, coupled with those presented below, further confirms that the CRD-BP and the cross-reacting p85 do not co-localize in cells and are functionally distinct proteins.

To determine if at least some CRD-BP is associated with ribosomal subunits, K562 cell polysomes were purified by centrifugation and then resuspended in a buffer containing 20 mM EDTA, which dissociates polysomes into ribosomal subunits and free mRNP. The EDTA-treated polysomes were then fractionated in a sucrose gradient. Each gradient fraction plus material in the pellet at the bottom of the tube were analyzed for ribosomal RNA content by gel electrophoresis and for CRD-BP by

immunoblotting. The small ribosomal subunits sedimented primarily in fractions 6-11, while the large subunits were in fractions 10-14 (Fig. 7, panels A and B). The CRD-BP co-sedimented with the subunits and was also
5 detected in the pelleted material, which is expected to contain undissociated polysomes and monosomes (Fig. 7C). Therefore, the CRD-BP co-fractionates with ribosomal subunits in K562 cells. The nature of the CRD-BP/subunit association is unclear. In view of the broad
10 fractionation range of the CRD-BP, we have not attempted to quantitate relative CRD-BP levels from one fraction to the next.

Data from gel retardation and RNA-protein binding experiments indicate that p85 does not bind to the c-myc
15 CRD RNA (31, 32). Fig. 7C also shows that the small portion of p85 that does co-pellet with polysomes is not bound to the dissociated ribosomal subunits. Rather, it sediments at the top of the gradient (Fig. 7C). Similar results were obtained using crude cytoplasmic lysate
20 (S20) treated with EDTA (data not shown). In summary, p85 reacts with polyclonal anti-CRD-BP antibody but does not bind to c-myc CRD RNA (30, 31) and does not co-fractionate with the CRD-BP in cell lysates.

To verify the association of the CRD-BP with
25 ribosomal subunits using an independent method, immunoprecipitation (IP) experiments were performed using P protein antibodies, which react specifically with proteins associated with the large (60S) subunit. K562

cell polysomes were dissociated into subunits in the presence of 20 mM EDTA and IP'd with anti-P antibody serum or normal human serum. The IP'd proteins were then analyzed by immunoblotting using antibodies against the P-proteins and the CRD-BP. The anti-P protein antibodies IP'd the three 60S proteins (P_0 , P_1 , and P_2), as expected (Fig. 8A, lane I). None of these proteins were IP'd by normal human serum (lane N). The anti-P protein antibodies also IP'd the CRD-BP (Fig 8B, lane I). These findings confirm that the CRD-BP is associated with ribosomal subunits in K562 cell extracts.

C. Discussion

The CRD-BP is thought to stabilize c-myc mRNA by shielding its coding region from endonucleolytic attack (22, 30, 31). In this respect, it might be similar to the iron response protein that binds to and protects the 3'-UTR of transferrin receptor mRNA (reviewed in 41). However, the CRD-BP differs from the iron response protein and from many other mRNA-binding proteins in at least two ways. (i) Most such proteins bind within the 3'-UTR, while the CRD-BP binds to the c-myc mRNA coding region. It does not bind *in vitro* to RNA substrates from either of the c-myc untranslated regions (30). The coding region of c-fos mRNA also contains an mRNA half-life determinant that is a protein-binding site (42). Perhaps the function of the myc and fos mRNA coding region determinants and their respective binding proteins is related to the regulation of myc and fos

protein expression. (ii) The *c-myc* CRD-BP is developmentally regulated, being expressed abundantly in fetal and neonatal life but not in adult animals (32). Perhaps the CRD-BP has a special role in embryonic/fetal development.

The CRD-BP contains four KH domains and an RGG box, and it co-fractionates with polysomes and ribosomal subunits. These findings are consistent with it being an RNA-binding protein whose function is related in some way to translation and/or mRNA metabolism. The CRD-BP also co-fractionates with ribosomes in the absence of *c-myc* mRNA (Fig. 5). Perhaps it is bound both to *c-myc* mRNA and to ribosomes in intact cells. If so, it might be carried along with the translating ribosomes as a reservoir to be used when needed to bind to any unprotected *c-myc* mRNA molecules. The CRD-BP also contains a putative nuclear localization sequence and two putative nuclear export sequences (Figs. 1 and 2). We do not know if the CRD-BP shuttles between the nucleus and the cytoplasm. If it does shuttle, however, it appears to spend most of its time in the cytoplasm of growing cells, because little of it is detected in the nucleus at steady-state (Fig. 6).

Consistent with the unique features of the CRD-BP noted above, the CRD-BP and hKOC protein appear to represent a unique subfamily of KH domain-containing RNA binding proteins. Other putative RNA-binding proteins, including the FUSE-binding protein, P-element somatic

inhibitor, and *C. elegans* M88.5, resemble the CRD-BP in containing four KH domains (43). However, several structural features of these proteins distinguish them from CRD-BP and hKOC. The KH domains of the P-element somatic inhibitor and FUSE-binding proteins are located toward their amino termini and are organized as an evenly-spaced, four unit repeat. These proteins also contain either glycine rich or glutamine-rich stretches in their amino and carboxy termini. The overall organization of the four KH domains of M88.5 is most similar to CRD-BP and hKOC. It contains two pairs of KH domains separated by 83 amino acids. However, in contrast to CRD-BP and hKOC, the amino terminus of M88.5 is glutamine-rich and lacks an RGG box. The FUSE-binding protein contains a sequence resembling an RGG box, but this sequence is located between the third and fourth KH domains, which is not the case for the CRD-BP and hKOC protein. Finally, the core sequences of the KH domains of these other proteins are very different from those in either CRD-BP or hKOC.

Several structural and functional similarities are also noted between the CRD-BP and the FMRP, the protein encoded by the FMR1 gene, mutations in which are responsible for the most common form of inherited mental retardation (44, 45). Both proteins contain KH domains and an RGG box (37, 38) as well as nuclear import and export signals (39). Both proteins associate with ribosomes and probably with mRNA as well (33, 49, 46).

Neither protein is required for cell viability, because individuals who fail to express FMRP survive, while perfectly normal adult animals do not express the CRD-BP at levels detectable by immunoblotting and/or gel retardation assays (32). There are also some significant differences between FMRP and CRD-BP, particularly in their expression patterns. Both are expressed abundantly during fetal life, but only FMRP is detected in adult tissues (47-49).

10 The structural features of the CRD-BP and its developmental regulation pattern suggest that it might be an oncofetal protein, for the following reasons: (i) It is expressed abundantly only in fetal and neonatal life (32). (ii) All of the mouse CRD-BP EST's that are
15 currently in the database are derived from either fetal tissue or from cell lines, including embryonic stem cells. These include AA073173 (from 13 day old embryonic heart tissue), AA619650 and AA399833 (from a pre-implantation blastocyst), AA073514 (from the P19
20 embryonic carcinoma cell line treated with retinoic acid), and D76662 and D76781 (from the F9 embryonic carcinoma cell line). (iii) The CRD-BP is expressed in many cell lines, all of which are neoplastic or pre-neoplastic. It is expressed at high levels in K562,
25 HeLa, and 3T3 cells (Figs. 3 and 4 and data not shown) and at low levels in other lines such as HL60, a human promyelocytic leukemia cell, and H4IIE, a rat hepatoma cell (data not shown). (iv) It is similar but not

identical to the hKOC protein that is overexpressed in
pancreatic cancer and in some other tumors (Fig. 2 and
ref. 40). If the CRD-BP is an oncofetal protein, it
would join a growing list of RNA-binding proteins that
5 influence the early development of the organism and/or
that affect carcinogenesis. For example, mutations in
the Elav proteins influence *Drosophila* development
(reviewed in 50-53), while mutations in other RNA-binding
protein genes result in male infertility or mental
10 retardation (44).

D. Detecting the CRD-BP in Clinical Samples

Human tumor tissues were provided by physicians and
surgeons at the UW-Madison Clinical Cancer Center. The
tissues were homogenized, and a crude cytoplasmic extract
15 was prepared. The extract was then fractionated by two-
dimensional gel electrophoresis at Kendrick Laboratories
(Madison, WI). Following electrophoresis in the second
dimension, the proteins were transferred to PVDF
membranes and returned to our laboratory.

20 CRD-BP was visualized by incubating the membranes
with antibodies to mouse CRD-BP. These antibodies cross-
react with human CRD-BP.

Findings are as follows:

1. We detect abundant CRD-BP in human breast
25 cancer, colon cancer, and pancreatic cancer tissues. We
expect to find similar results with other non-hemopoietic
cancers.

2. A significantly smaller amount of CRD-BP is detected in one normal human breast tissue sample.

3. No CRD-BP is detected in several human leukemia samples.

5 Our conclusion from these studies is that the CRD-BP is overexpressed in non-leukemia human carcinomas.

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